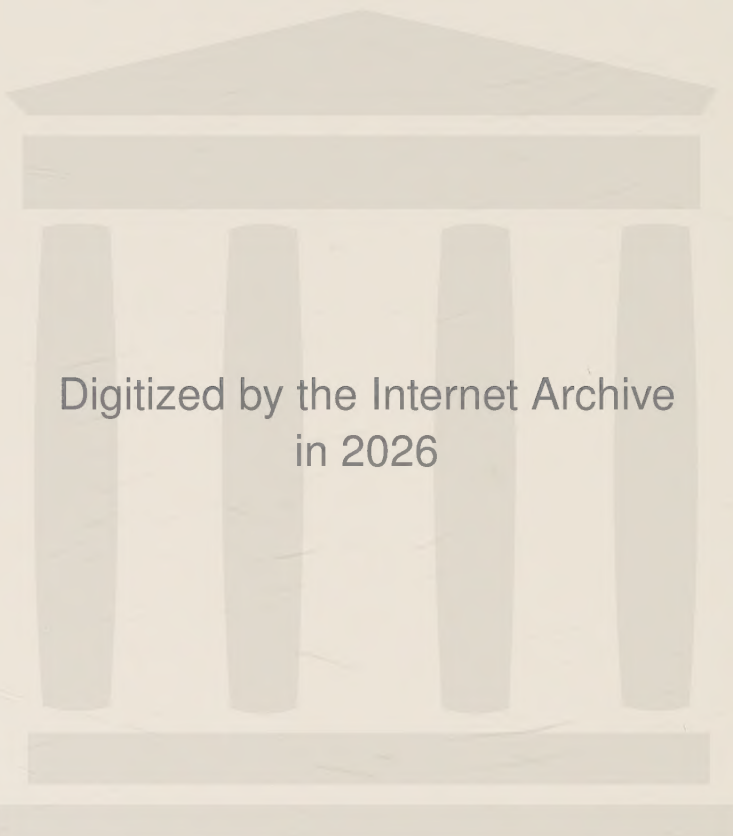


**Quantitative determination of tocopherols
with special reference to
the vitamin E requirements of low birth weight infants**

Lennart Jansson

Hund 1981

Malmö 1981



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QUANTITATIVE DETERMINATION OF TOCOPHEROLS
WITH SPECIAL REFERENCE TO
THE VITAMIN E REQUIREMENTS OF LOW BIRTH WEIGHT INFANTS

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This thesis is based on the following publications:

- I Nilsson B, Johansson B, Jansson L and Holmberg L.
Determination of plasma α -tocopherol by high-performance liquid chromatography.
J Chrom 145:169-72, 1978.
- II Jansson L, Nilsson B and Lindgren R.
Quantitation of serum tocopherols by high-performance liquid chromatography with fluorescence detection.
J Chrom 181:242-47, 1980.
- III Jansson L, Holmberg L, Nilsson B and Johansson B.
Vitamin E requirements of preterm infants.
Acta Paediatr Scand 67:459-63, 1978.
- IV Jansson L, Akesson B and Holmberg L.
Vitamin E and fatty acid composition of human milk.
Am J Clin Nutr 34:8-13, 1981.
- V Jansson L, Holmberg L and Jakobsson I.
The effects of dietary γ -tocopherol on serum tocopherols in formula fed infants.
Acta Paediatr Scand. In press 1981.

In the text the publications will be referred to by their Roman numerals.

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INTRODUCTION

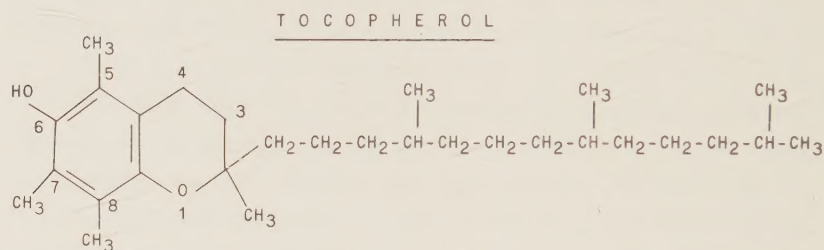
Historical

In 1922 Evans and Bishop found lettuce, wheat germs and dried alfalfa leaves to contain a fat soluble substance that prevented fetal death and resorption in rats brought up on a rancid lard diet. This substance was later called vitamin E (Sure 1924). In 1927 Evans prepared a potent vitamin E concentrate from wheat germ oil and after consulting G Calhoun (professor of Greek) named the substance tocopherol (Evans et al 1936) after the Greek words tokos (offspring) and pherein (to bear). The ending -ol was added to indicate that the substance was an alcohol. In the 1930s four tocopherols were isolated and synthesised. They were named α -, β -, γ - and δ -tocopherol. During the following three decades four related compounds: α -, β -, γ - and δ -tocotrienol were isolated and synthesised. The last substance, δ -tocotrienol, was synthesised by Whittle et al in 1966.

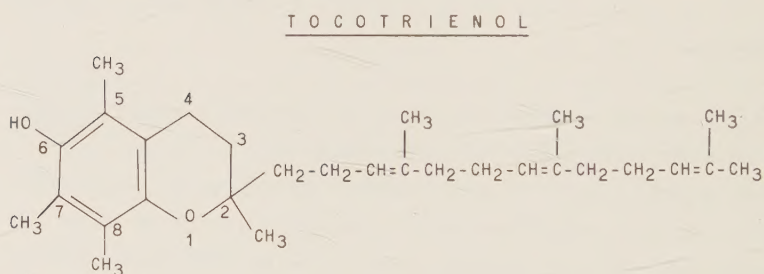
Chemistry

Vitamin E occurs in green plants. Eight naturally occurring substances with vitamin E effect are known, viz. α -, β -, γ - and δ -tocopherol and α -, β -, γ - and δ -tocotrienol (Fig. 1). The ring structure with a saturated phytol side-chain is called tocol 2-methyl-2(4', 8', 12'-trimethyl-tridecyl) chroman -6-ol. The structure of tocol is common to all the four tocopherols, which differ from one another by the number of methyl groups in the ring structure. The tocopherols have three asymmetric carbon atoms located at C-2, C-4' and C-8' (Fig. 1). There is thus a possibility for different optical isomers. The only naturally occurring stereoisomer of α -tocopherol is *d*- α -tocopherol or 2R-(4'R, 8'R) -5, 7, 8-trimethyltolcol. Synthetic preparations

are ι - α -tocopherol (2S, 4'R, 8'R) and mixtures of the d - and ι -forms: dl - α -tocopherol. The name dl - α -tocopherol is unsatisfactory since it denotes two different substances: 2-*ambo*- α -tocopherol - a mixture of d - and ι -forms, and *all-rac*- α -tocopherol, a racemic mixture of all eight possible optical isomers in the form of 4 racemates. The tocotrienols differ from the tocopherols by having three double bonds in the phytyl-chain (Fig. 1).



- α - Tocopherol (5,7,8 - trimethyltolcol)
 β - Tocopherol (5,8 - dimethyltolcol)
 γ - Tocopherol (7,8 - dimethyltolcol)
 δ - Tocopherol (8 - methyltolcol)



- α - Tocotrienol (5,7,8 - trimethyl tocotrienol)
 β - Tocotrienol (5,8 - dimethyl tocotrienol)
 γ - Tocotrienol (7,8 - dimethyl tocotrienol)
 δ - Tocotrienol (8 - methyl tocotrienol)

Fig. 1. Chemical structure of naturally occurring tocopherols and tocotrienols.

Definition

"Vitamin E should be used as the generic descriptor for all tocol and tocotrienol derivatives exhibiting qualitatively the biological activity of α -tocopherol" (International Union of Nutritional Sciences 1976). The term tocopherol should be used as a generic descriptor for all mono-, di- and tri-methyl tocols irrespective of their biological activity. Since the tocotrienols possess vitamin E activity, the term tocopherol is not synonymous with vitamin E.

METHODS OF ASSAY

Biological assay; Biological activity

The biological activity of the various vitamin E compounds is assessed with so-called bioassays. In these assays a deficiency state is induced in a group of experimental animals, after which the effect of administration of a substance with unknown vitamin E activity is estimated. The most widely used biological assays are the resorption-gestation assay of female rats (Mason and Harris 1947), and the prevention of necrotic liver degeneration in rats (Century and Horwitt 1965). Evaluation of the vitamin E activity with bioassays is, however, difficult because the various vitamin E compounds differ in absorption and retention patterns between different organs. Further, the results often vary with the general composition of the diet and the species of the experimental animals used (Horwitt 1975).

The biological activity of the various vitamin E compounds are thus difficult to define. This explains the occasional changes in the choice of standard substance. Up to 1956 the standard was 2-*ambr*- α -tocopheryl acetate, after which it was *all-rac*- α -tocopheryl acetate, both of which designate *dl*- α -tocopheryl acetate (see Chemistry). One milligram of *all-rac*- α -

tocopheryl acetate is defined as 1.0 international unit (I.U.) of vitamin E, and the combined vitamin E effect of different tocopherols has been expressed in I.U. In 1976 the reference substance was again changed (IUNS 1976). According to these last-mentioned recommendations, *d*- α -tocopherol should be used as standard, and the combined vitamin E content in food products, for example, should be given in milligrams of *d*- α -tocopherol equivalents.

Chemical assay

Spectrophotometric method. Most chemical assays are based on the so-called Emmerie-Engel reaction (Emmerie and Engel 1938). This reaction is based on the fact that when tocopherol reduces ferric to ferrous iron in the presence of α , α' -dipyridyl (a bathophenanthroline) a red complex is formed, which can be measured spectrophotometrically at 520 nm. This method measures the total tocopherol content. The various vitamin E compounds can thus not be determined separately.

Thin layer chromatography (TLC). With TLC it is possible to separate the tocopherols and then determine them separately with the Emmerie-Engel reaction (Bieri and Prival 1965). But the method has the disadvantage of relatively low recoveries making it difficult to use at low concentrations of tocopherol (Horwitt et al 1968).

Gas-liquid chromatography (GLC). With GLC the different tocopherols can be separated and the recoveries are generally better than with thin layer separation (Lehman and Slover 1971). But it is time-consuming because the tocopherols must first be converted to volatile compounds.

High performance liquid chromatography (HPLC). HPLC has been elaborated during the last decade and for various purposes it has proved superior to GLC and TLC.

This work describes new HPLC methods for determining α ,- β ,- γ - and δ -tocopherol separately in serum and in milk.

FUNCTION OF VITAMIN E

Deficiency diseases in animals

Vitamin E is necessary for normal development of various organ systems in different animal species. It was Evans and Bishop (1922) who discovered that vitamin E is necessary for normal fetal development in the rat. This observation was followed by the discovery of other vitamin E deficiency diseases in several other sorts of animals (Table 1).

Table 1. Vitamin E deficiency diseases in various species of animals.

Disease	Animal species	Prevented by		
		Vitamin E	Antioxidant	Selenium
Embryonic degeneration	rat	X	X	
Encephalomalacia	chick	X	X	
Necrotic liver degeneration	rat	X		X
	pig	X		X
Muscular dystrophy	lamb	X		X
	calf	X		X
	pig	X		X
	horse	X		X
	turkey	X		X
Exudative diathesis	chick	X		X

Common to all of these diseases is that they can be prevented by administration of vitamin E. Several of the above conditions can, however, also be controlled by treatment with synthetic anti-oxidants and selenium (Table 1). Examples of vitamin E deficiency diseases that can be prevented by an adequate supply of a synthetic anti-oxidant, such as diphenyl-paraphenylene diamine, are encephalomalacia in chicks and embryonic degeneration in the rat. Examples of the prevention of vitamin E deficiency diseases by selenium, so-called vitamin E and selenium deficiency diseases (Lannek and Lindberg 1975) are necrotic degeneration of the liver in rats and pigs, muscular dystrophy in lambs, calves, pigs, horses and turkeys, as well as exudative diathesis in chicks (Table 1). It would thus appear that vitamin E has two functions in the organism; that of a general antioxidant and that of a more specific antioxidant in association with selenium metabolism.

Anti-oxidant theory

Vitamin E is a strong anti-oxidant in vitro (Olcott and Mattill 1941). The discovery that vitamin E deficiency in various animals can be controlled by treatment with a synthetic antioxidant has given rise to much speculation as to whether vitamin E has an anti-oxidative capacity also in vivo. This so-called general antioxidant theory has been summarized by Tappel (1972). The background to this theory is the discovery of so-called free radical lipid peroxidation. These reactions are well defined in vitro and are believed to be of significance in vivo (Pryor 1973).

A simplified scheme of free radical lipid peroxidation has been given by Chow (1979):

- (a) $RH \rightarrow R\cdot$
- (b) $R\cdot + O_2 \rightarrow ROO\cdot$
- (c) $ROO\cdot + RH \rightarrow ROOH + R\cdot$
- (d) $R\cdot + AH \rightarrow RH + A\cdot$

Destruction of unsaturated fatty acids (RH) leads to formation of free radicals ($R\cdot$) (a), fatty acid peroxy radicals ($ROO\cdot$) (b) and unsaturated hydroperoxides ($ROOH$) (c). Ferrous iron catalyzes these reactions and consequently accelerates the lipid peroxidation (McKnight 1965, Wills 1965, Wills 1966). These reactions can be interrupted by administration of an anti-oxidant (AH) (d).

What precipitates these reactions, i.e. the cause of the first reaction (a) is not known. According to one theory, it is initiated by formation of the superoxide anion (O_2^-), (Misra and Fridovich 1972, Misra 1974). But the formation of fatty acid peroxy radicals and hydroperoxides is not only a disadvantage for the organism, for these substances are necessary for the bactericidal activity of neutrophil granulocytes and are normal intermediary substances in prostaglandin synthesis (Chow 1979).

Interaction with selenium

It has long been known that selenium can control a variety of diseases due to vitamin E deficiency, such as muscular dystrophy in lambs, calves, pigs and young horses as well as exudative diathesis in chicks (Table 1), but the reason why was not known until the 1970s. Rotruck et al (1973) discovered that selenium is a constituent of the enzyme glutathione peroxidase. This enzyme protects cellular and subcellular membranes from peroxidative injury. Glutathione peroxidase (GP) catalyses the reduction of hydrogen peroxide to water:



The glutathione disulfide (GSSG) (oxidized glutathione) is then reconverted to reduced glutathione (GSH) by glutathione reductase (GR).:



The NADPH used in this reaction is generated via the hexose monophosphate shunt.

VITAMIN E IN HUMAN NUTRITION

Vitamin E has been tried in the treatment of numerous human diseases. Vitamin E deficiency in humans is, however, still a controversial subject with the exception of vitamin E deficiency in association with malabsorption syndromes and in premature neonates (Bieri and Farrel 1976). In this review only the vitamin E requirements of preterm infants will be discussed.

The premature infant is born with low body stores of vitamin E. Dju et al (1952) found that the body stores of tocopherol in human fetuses and in stillborn fullterm infants increased with bodyweight. Wright et al (1951) showed that in newborn infants the serum level of tocopherol was about one-fifth of that in the mothers, which indicates a limited placental transfer of vitamin E.

No association was known between this finding and any particular disease until Oski & Barness (1967) found it to be related to haemolytic anaemia in premature infants. They described 11 infants who had vitamin E deficiency and haemolytic anaemia and in whom administration of vitamin E corrected the abnormality, as judged from the serum tocopherol level, peroxide haemolysis test, haemoglobin level and reticulocyte count. Other investigators have obtained somewhat different results. Lo et al (1973) found administration of vitamin E to have a favourable effect on the development of haemoglobin in formula fed infants born before term. Still the findings of other investigators did not agree with the observation of Oski and Barness. Thus,

Goldbloom and Cameron (1963) and Panos et al (1968) found no significant effect of supplementary vitamin E on blood parameters in preterm infants.

The most comprehensive investigations of vitamin E deficiency in preterm infants are those made by Gross and Melhorn (1971a, 1971b). Their investigations led to a better understanding of the complex mechanisms governing the vitamin E requirements in preterm infants. In a large material they examined the vitamin E requirement in relation to the dietary supply of iron as well as gestational age relative to the absorption of vitamin E. They found significantly lower haemoglobin levels and higher reticulocyte counts in 6-10 week old preterm infants with a birthweight below 2,000 g and a gestational age of less than 36 weeks when the iron was given without extra vitamin E. They also found a lower absorption of vitamin E during the first 8 weeks of life in preterm infants with a gestational age of less than 32 weeks.

In a later investigation Gross and Melhorn (1974) showed the advantage of a water-soluble vitamin E preparation, *d*- α -tocopheryl polyethylene glycol 1,000 succinate (TPGS), which was absorbed better than the fat soluble *d*- α -tocopheryl acetate.

Besides the iron intake the amount of polyunsaturated fatty acids (PUFA) in the diet is the most important factor governing the vitamin E requirements. Hassan et al (1966) showed that preterm infants fed a diet high in PUFA and low in vitamin E developed a syndrome comprising oedema, thrombocytosis, skin changes and morphological changes in the erythrocytes. These abnormalities disappeared on treatment with vitamin E.

The combined effect of supplementary iron and PUFA's on the vitamin E requirement in preterm infants has been studied by Williams et al (1975). They found that such infants brought up on a formula in which 32.5% of the fat consisted of linoleic

acid and given supplementary iron, developed signs of hyperhaemolysis. Supplementary iron had no demonstrable adverse effect on the blood picture when the amount of polyunsaturated fat in the formula was about 12%. Williams et al concluded that formula fed preterm infants did not require supplementary vitamin E even when supplementary iron was given provided that the polyunsaturated fat content of the formula was not too high. However, their conclusion regarding the administration of vitamin E is not applicable to Sweden, where even preterm infants are often fed human milk.

Breast milk contains more vitamin E than cow's milk (Quaife 1949). Therefore, the increase in serum tocopherol occurs much earlier in children brought up on breast milk (Wright et al 1951). This had induced manufacturers to increase the amount of vitamin E in modern formulae based on cow's milk. It is, however, not properly known how the composition of tocopherol in human milk varies during lactation. Neither is it known exactly whether or how the composition of tocopherol varies with the fat content or with the composition of the fatty acids. The fatty acid composition of breast milk varies with the maternal fat consumption. Söderhjelm (1953) found that the polyunsaturated fat content of breast milk increased if the mother's diet was high in polyunsaturated fat. Insull and Ahrens (1959) found linoleic and linolenic acid to constitute 45% of the total fatty acids in breast milk of a woman with a high consumption of corn oil.

Another important point is the quality of the supplemented vitamin E. Of the naturally occurring tocopherols in food products, α - and γ -tocopherols are the most important. In animal fats α -tocopherol constitutes over 90% of the vitamin E content, but in vegetable oils, such as soybean oil and corn oil, γ -tocopherol is the predominant tocopherol.

The various tocopherols differ in biological activity. Thus, α -tocopherol is regarded as the most active and ten times as

active as γ -tocopherol (Bieri and Evarts 1974). Complete calculation of the vitamin E content of food products must therefore comprise chemical separation and determination of each of the most important tocopherols.

PRESENT INVESTIGATION

THE PURPOSES OF THIS INVESTIGATION WERE:

- 1) to devise a method for determining α -, β -, γ - and δ -tocopherol separately in serum as well as in milk, and with the aid of such a method;
- 2) to assess the requirement of supplementary vitamin E in low birth weight (LBW) infants;
- 3) to estimate the relation between the different tocopherols, the fat content and the fatty acid composition in human milk; and
- 4) to study the effect of dietary vitamin E on the serum tocopherols in infants.

MATERIALS

Patients (paper III)

The clinical material consisted of 57 low birth weight (LBW) infants born at Malmö General Hospital in 1975-1977. Thirtyfour had a birthweight (BW) < 2,000 g or a gestational age (GA) < 35 weeks, 23 BW > 2,000 g. Except for two infants with mild respiratory distress syndrome, none had any detectable sign of disease during the study period. The infants were examined haematologically within the first 24 hours and at 8-10 weeks of age. Blood for tocopherol determination was obtained by venipuncture.

Study groups (III)

The infants in each BW-GA-category were randomly assigned to one or another of three groups given the following therapeutic regimens:

1. Ferrous succinate (2-3 mg/kg/day) from 3 weeks of age.
2. Iron as group 1 plus α -tocopheryl acetate (15 mg/day) from 10 days of age.
3. α -Tocopheryl acetate as in group 2, but iron not started until 10 weeks of age.

Dietary arrangements (III)

All infants were given human pasteurized milk for the first two weeks or until they weighed > 2,100 g. The milk was then replaced by the commercial formula Milkotal[®] (AB Findus). From 10 days of age daily treatment with vitamins was started: Protovit[®] (Roche) 0.5 ml/day and tetrahydrofolate (Leucovorin[®] Lederle) 50 μ g/day.

Patients, study groups, dietary arrangements (paper V)

The clinical material consisted of 20 infants, aged 3-15 months. All had received a cow's milk protein-free diet for at least 2 months because of suspected intolerance to cow's milk. Ten infants were fed a diet based on Nutramigen[®] (Mead Johnson) and 10 a diet based on SojaSemp[®] (AB Semp). Blood samples for determination of tocopherol were obtained by venipuncture, and the sera were separated and stored at -20°C until analysed.

Breast milk samples (paper IV)

Forty human milk samples were collected from 40 healthy mothers

in different stages of lactation. Six were colostrals samples obtained before the early morning feed. Ten transitional and 24 mature milk samples consisted of residual milk collected after the regular feed to the baby during a 2 day period. The milk was frozen and stored at -20°C until analysed.

METHODS

Determination of serum α -tocopherol (I, III)

Serum α -tocopherol was determined with a new HPLC method described in detail in paper I. The equipment was a Waters model ALC/GPC 204 liquid chromatograph, consisting of a model 6000 pump, a U6K injector, a model 440 UV spectrophotometer. The column (Waters Corasil I) was eluted with n-hexane-diisopropylether (96:4). The absorbance at 280 nm was recorded.

Preparation of samples

To each plasma (or serum) sample (200 μl) was added an equal volume of 99.5% ethanol containing the internal standard. After the addition of 200 μl n-hexane, the sample was cyclomixed. After centrifugation, 15 μl of the organic layer was injected directly into the column.

Determination of α -, β -, γ - and δ -tocopherols (II, IV, V)

The various tocopherols in serum, human milk and formulae were determined with a HPLC-method described in detail in paper II. The equipment consisted of Waters model 6000 pump, a U6K injector, and a Waters μ -Porasil column. The column was eluted with n-hexane-diisopropylether (92:8), and the fluorescence

intensity of the column eluent was monitored by a Schoeffel variable wavelength spectrofluorimeter equipped with a deuterium lamp. The excitation wavelength was set at 295 nm and a cut-off emission filter of 370 nm was used.

Preparation of samples: To each serum sample (500 μ l) was added an equal volume of 99.5% ethanol containing the internal standard *dl*-tocol. After the addition of 1 ml n-hexane, the sample was cyclomixed, centrifuged, and the organic layer pipetted off and evaporated to dryness in a stream of nitrogen. When dry the residue was redissolved in 50 μ l n-hexane, and 10-20 μ l was injected into the column.

Total lipid and fatty acid determination (V)

Total lipid content was determined gravimetrically after extraction with chloroform/methanol (1:1).

Fatty acid composition was analysed by gas chromatography as fatty acid methyl esters (Åkesson et al).

RESULTS (I-V)

I. Determination of plasma α -tocopherol by high-performance liquid chromatography

Standard solutions: Eight standard mixtures with a varying content of α -tocopherol and a constant content of α -tocopheryl acetate (the internal standard) were prepared and analysed by HPLC). The plot of peak area ratios against concentration for the components in the calibration mixtures showed good linearity.

Chromatogram: The peaks of α -tocopherol and the internal standard were well separated, and the retention times were 5.1 and 3.3 min, respectively.

Recovery: The recovery of α -tocopherol added to serum samples was 100%.

Reproducibility: This was tested by injection of extracts from the same plasma sample three times a day over a period of three days. The coefficient of variation was 6%.

Sensitivity: This was tested by injecting pure α -tocopherol. The minimum detectable amount was 84 pmol.

Normal values: α -tocopherol in plasma from 14 healthy adults ranged from 17 to 39.9 $\mu\text{mol/l}$.

II. Quantitation of serum tocopherols by high performance liquid chromatography with fluorescence detection.

Standard solutions: Four standard mixtures with increasing concentrations of α ,- β ,- γ - and δ -tocopherol and a constant concentration of the internal standard *dL*-tocol were prepared and analysed by HPLC. The fluorescence peak area ratios plotted against concentration showed a good linearity for all tocopherols.

Chromatogram: The tocopherols and *dL*-tocol were well separated. The retention times relative to *dL*-tocol were for α : 0.37, β : 0.51, γ : 0.59 and δ : 0.86.

Recovery: The recoveries of pure tocopherols added to a serum sample were 92% for δ -tocopherol and 100% for α ,- β - and γ -tocopherol.

Reproducibility: This was tested by analysing extracts from two different serum pools twice a day over a period of 5 days. The coefficient of variation were for α -, β - and γ -tocopherol: 1.5%, 22% and 7%, respectively.

Sensitivity: The minimal detectable amount of pure α -tocopherol was 21 pmol.

Normal values: The concentrations of α -, β - and γ -tocopherol in the sera from 10 normal individuals, 15 mothers and their infants (cord blood) are presented in detail in paper II.

III. Vitamin E requirements of preterm infants

This study was concerned with the effect of supplementary vitamin E on the haemoglobin concentration and the reticulocyte and platelet counts in LBW infants. The absorption of supplementary vitamin E was assessed from determinations of the serum α -tocopherol with HPLC (I). Under the prevailing dietary conditions supplementary α -tocopheryl acetate in a dose of 15 mg/day significantly raised the serum α -tocopherol. All the infants given supplementary vitamin E had serum α -tocopherol levels above 11.6 $\mu\text{mol/l}$ (0.5 mg/dl) at 4-5 weeks. Infants who had birthweights of at most 2,000 g or a gestational age of at most 35 weeks and who were treated with supplementary vitamin E had significantly higher haemoglobin and lower reticulocyte counts at 8-10 weeks of age than those not given supplementary vitamin E. No such difference was demonstrable in infants with a birthweight of more than 2,000 g. When 16.5 mg α -tocopheryl acetate was given daily from 10 days of age no signs of hyperhaemolysis appeared, even when supplementary iron, 2-3 mg/kg/day, was given from 3 weeks of age.

IV. Vitamin E and fatty acid composition in human milk

The composition of vitamin E, the total fat content and the fatty acid composition were determined in 40 samples of human milk obtained in different stages of lactation. The total tocopherol content was significantly higher in the specimens obtained in the early stages of lactation than in samples of mature milk. This difference was due to a high α -tocopherol content of the colostrum and transitional milk.

The total fat content was lower in colostrum than in the transitional and mature milk. Also the fatty acid composition of the colostrum milk differed in some respects from the other types of milk in that it had a lower lauric acid level and higher levels of, above all, the long-chained fatty acids, arachidonic acid (20:4;n-6) and docosahexaenoic acid (C22:6;n-3).

The relation between vitamin E and fat was expressed as the total tocopherol/lipid ratio and the total tocopherol/linoleic acid ratio. Both these ratios were higher in colostrum and transitional milk than in the mature milk. In colostrum and transitional milk the total tocopherol/linoleic acid ratios exceeded 1 I.U./g, but in mature milk this ratio was significantly lower (<1 I.U./g in 9 out of 24 mature milk samples). A positive correlation was found between tocopherol and total fat and between tocopherol and linoleic acid in mature milk.

V. The effects of dietary γ -tocopherol on the serum tocopherols in formula fed infants

The vitamin E composition was determined with HPLC (II) in two different formulae, Nutramigen[®] and SojaSemp[®]. These differed from each other mainly in respect of the γ -tocopherol content, which was, on the average, 22.8 $\mu\text{mol/l}$ in Nutramigen[®]

and $3.6 \mu\text{mol/l}$ in SojaSemp[®]. The higher content of γ -tocopherol in Nutramigen is due to its high concentration of corn oil. Whether or how this difference in vitamin E composition between the two formulae affected the serum tocopherols was investigated in two groups of infants, one fed Nutramigen[®] and one fed SojaSemp[®]. No difference in serum total tocopherol levels was found between the two groups. But the groups did differ in the relative amounts of tocopherol. In the Nutramigen group γ -tocopherol constituted, on the average, 16.7% of total tocopherol against only 4.2% in the SojaSemp group. The differences were greatest in the smallest infants fed only formula.

GENERAL DISCUSSION

Study I describes a new method for determining α -tocopherol in blood samples. Since the extraction is not preceded by saponification, and since the specificity for α -tocopherol is high it is superior to the methods hitherto available (colorimetry, TLC, GLC). A disadvantage of the method is the choice of internal standard, α -tocopheryl acetate, which excludes its use in the analysis of vitamin E in food products supplemented with α -tocopheryl acetate. However, we found that the method could be successfully used in the analysis of serum samples even when the patients had been given α -tocopheryl acetate orally. This was shown by analysis of serum samples without the internal standard. In such sera no α -tocopheryl acetate could be detected. The acetate ester is hydrolysed in the digestive tract and is absorbed as free tocopherol (Blomstrand and Forsgren 1968). But the choice of the internal standard makes the first described method unsuitable if the patient has previously been treated parenterally with α -tocopheryl acetate. Such injections have been used to raise the serum vitamin E levels quickly in immature infants (Graeber et al 1977). Besides this, we found that with UV detection the sensitivity and specificity were too low for recognition of other tocopherols in the serum (β - and γ -tocopherol) (Bieri and Prival 1965, Chow 1975). Leenher et al (1979) used UV detection also in the analysis of the non- α -tocopherols in serum samples, but we have not been able to reproduce their results. However, as reported by Abe et al (1975), with the aid of the fluorescence technique, it is possible to increase the sensitivity of the method and make it more specific. But it is important to record also the UV absorbance if one wishes to demonstrate the α -tocopheryl acetate occurring in many food products, for example, since α -tocopheryl acetate does not fluoresce under the same conditions as the "free" tocopherols.

The second publication (II) describes a method for determining the various tocopherols separately by HPLC with fluorescence detection. The method is quick and specific and can be used (by simultaneous UV-detection) also for analysis of α -tocopheryl acetate. It does not require saponification of the samples and thereby avoids the risk of loss of tocopherol. Since the method uses *dL*-tocol as internal standard it is also better than the method described in paper I because it can be used in the analysis of serum samples obtained even after parenteral administration of α -tocopheryl acetate. Both HPLC methods (I and II) are more sensitive than the hitherto available TLC method (Horwitt 1968).

A number of factors must be taken into account when calculating the vitamin E requirements of a given individual. The main factors determining the vitamin E requirements are the dietary iron and polyunsaturated fat content of the diet. The biological activity of the vitamin E given is also important. The significance of these variables is discussed in papers III-V, especially the nutritional implications for the preterm infant.

The third study (III) concerns mainly the tocopherol requirement in LBW infants relative to the composition of their diet. We found that administration of α -tocopheryl acetate in a dose of 16.5 mg/day significantly raised the serum level of α -tocopherol. Even in the most premature infants with a gestational age of less than 32 weeks the serum α -tocopherol exceeded 11.6 $\mu\text{mol/l}$ (0.5 mg/dl) (the critical lowest level according to previous studies) at 4-5 weeks. This contrasts with Gross and Melhorns' finding of reduced absorption of α -tocopheryl acetate in infants with a birthweight < 1 500 g and a gestational age of < 32 weeks up to the 8th to 10th week of life. The uncertainty about the absorption of oral vitamin E induced certain workers to give α -tocopherol or α -tocopheryl acetate parenterally. Such treatment has proved to lower the

carboxyhaemoglobin (Gross et al 1977) and the bilirubin levels (Dyggve and Probst 1963, Gross 1979) in LBW infants. Parenteral treatment of the type given by Gross et al might also have a favourable effect on the occurrence of bronchopulmonary dysplasia (Ehrenkrantz et al 1978) and retrolental fibroplasia (Johnsson et al 1974). But, like us, Bell et al (1979) found the absorption of α -tocopherol in premature infants to be considerable after oral treatment with vitamin E.

Using a dietary regimen resembling that in the present investigation (III), Lo et al (1973) found no signs of haemolysis as long as the infants were fed human milk, but only after the beginning of formula feeding. However, the formula used by Lo et al had a lower vitamin E content (0.4 mg/l) than those now generally used. The concentrations of PUFA and Fe were not stated and could therefore not be compared with our results. Neither did Lundström et al (1977) observe any signs of vitamin E deficiency in children brought up on human milk alone in spite of the fact that supplementary iron (2 mg elemental iron/kg/day) was started at 2 weeks of age. However, also supplementary vitamin E was given (5 I.U./day) from 2 weeks of age.

That supplementary iron is an important factor governing the vitamin E requirements of preterm infants was shown by Melhorn and Gross (1971a). These workers found that administration of 8-10 mg Fe^{2+} /kg/day increased red cell haemolysis unless also supplementary vitamin E was given. Their investigation has been criticised because the amount of iron the infants received was so large. The dose was much larger than that now recommended (2 mg/kg/day); (American Academy of Pediatrics, Committee on Nutrition 1977). But a similar effect was also observed even after smaller doses of supplementary iron (2 mg/kg/day) when the diet was rich in polyunsaturated fatty acids (Williams et al 1975).

In our investigation (III) supplementary iron was given in a dose of 2-3 mg/kg/day to two groups of infants from the age of 3 weeks. The infants were fed human milk during the first few weeks or until they weighed 2,100 g. For practical reasons many of the infants were then fed an iron supplemented formula, which meant a further daily iron supply of 2 mg/kg. This in turn implied an unnecessary high intake of iron, but is a practice that is generally accepted in Sweden. Our investigation showed that under these circumstances supplementary α -tocopheryl acetate in a dose of 15 mg/day is to be recommended. The total iron supply could surely be reduced to 2 mg/kg/day without any risk of development of iron deficiency (AAP, Committee on Nutrition 1977). But it is not certain whether such a reduction in iron intake will make it possible to omit or reduce our recommended dose of vitamin E in preterm infants fed human milk. According to the latest American recommendations, also premature infants fed vitamin E enriched formulae should be given supplementary vitamin E in a dose of 5 I.U./day (AAP, Committee on Nutrition 1977).

Study IV concerns the vitamin E, fat and fatty acid composition in human milk. As pointed out previously, the vitamin E requirements varies with the intake of polyunsaturated fatty acids. We found that the vitamin E content of human milk varied during lactation, the total content of vitamin E being highest in colostrum and transitional milk and much lower in mature milk. One of the conclusions that can be drawn from our investigation is that so-called pooled human milk, which consists mainly of mature milk, gives the infant a smaller supply of vitamin E than do modern formulae, which contain about 10 I.U./l. It is thus possible that the difference in the tendency to haemolysis in our study III, compared with e.g. that by Williams et al (who used vitamin E enriched formula) was due at least partly to the fact that our infants received a smaller supply of vitamin E.

This is reflected in the relatively low vitamin E/linoleic acid ratios (mean 0.78, 9 of 24 samples below 0.67 mg α -tocopherol equivalents/g) that we found in the mature human milk. According to the American recommendations for formula fed LBW infants, the formula should contain 1 I.U. of vitamin E/g linoleic acid (0.67 mg β - α -tocopherol equivalents/g), besides which the infants should be given an extra daily supply of 5 I.U. vitamin E. The variation of the linoleic acid content of human milk, which depends to some extent on the mother's fat consumption, appears to be of less importance since the vitamin E content increased with the content of linoleic acid. However, on no occasion in our material did the linoleic acid constitute more than 18% of the total fatty acids in the milk samples. But if the mother consumes extremely large amounts of vegetable oils, the linoleic acid level in her breastmilk may be very high (Insull et al 1959).

A wide variety of formulae for newborn infants is now available for those who for some reason or other cannot be fed human milk. These formulae vary in composition. Their vitamin E content is usually given as the amount of total tocopherol (measured with the Emmeri-Engel method) or as α -tocopherol (often = the added amount of α -tocopheryl acetate). To check to what extent such a practice is justified we studied two different formulae containing no cow's milk protein, namely, Nutramigen[®] and SojaSemp[®] (V). We found that Nutramigen[®] contains the same amount of γ -tocopherol as the amount of α -tocopherol declared by the manufacturer. It is difficult to assess the biological effect of supplemented γ -tocopherol, but it is widely believed to be 10% of that of β - α -tocopherol (Bieri and Evarts 1974). We found that determination of only α -tocopherol in a formula can lead to underestimation of the actual vitamin E content, but also that determination of vitamin E as total tocopherol with conventional methods (Quaife 1947) can lead to overestimation of the amount of biologically active vitamin E. This applies, above all, to the analysis of formulae and diets rich in corn and soybean oil, both of which are rich in γ -tocopherol.

Yet, it is questionable whether the relative activity of γ -tocopherol given in the literature should be accepted without some reservation. Scott and Desai (1964) found that muscular dystrophy in chicks could be prevented by equal serum concentrations of α -, β - and γ -tocopherol. This suggests that all the tocopherols may have the same biological potency in the tissues. Whether this applies also to humans remains to be shown. At any rate, we found (paper V) γ -tocopherol to account for over 16% of the total serum tocopherol in infants fed a formula rich in γ -tocopherol. The serum γ -tocopherol level should therefore be determined when investigating the vitamin E status in humans, especially in those with a high intake of γ -tocopherol.

GENERAL SUMMARY

The present study may be summarized as follows:

- 1) New methods for determining α -tocopherol in serum samples and α -, β -, γ - and δ -tocopherol in serum samples and samples of human milk are described. These methods utilizing high performance liquid chromatography with either UV-detection or fluorescence detection or both, are quick and accurate and require only small samples.
- 2) Infants with a birthweight of $< 2,000$ g or a gestational age of < 35 weeks, fed pooled pasteurized human milk for the first few weeks, seem to benefit from supplementary vitamin E (15 mg α -tocopheryl acetate/day) given from 10 days of age.
- 3) Human milk varies in vitamin E-content. The early types of milk (colostral and transitional milk) contain more vitamin E than does mature milk. The predominant tocopherol

is α -tocopherol, especially in colostrum. The total tocopherol content of mature milk varies with the total lipid content as well as with the linoleic acid content. Still the vitamin E/linoleic acid ratio in mature milk expressed in mg α -tocopherol equivalents/g linoleic acid does often not comply with the recommendations for preterm infants.

- 4) The vitamin E content in infant formulae is best determined with methods that estimate the content of each of the various tocopherols (α -, β -, γ - and δ -tocopherol) separately. γ -Tocopherol is demonstrable in considerable amounts in serum from infants fed large amounts of it.

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APPENDIX

Papers I - V

Journal of Chromatography, 145 (1978) 169–172

Biomedical Applications

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CHROMBIO. 096

Note

Determination of plasma α -tocopherol by high-performance liquid chromatography

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Vitamin E, a fat-soluble vitamin, is chemically composed of four forms of tocopherol (α , β , γ , δ). α -Tocopherol is the most abundant member of the series (ca. 12–36 μ mole/l plasma) in human serum and is also biologically the most active form. A large number of methods [1–3] exists for the determination of tocopherols in plasma samples but most of these are based on indirect measurement of vitamin E. In such methods carotenoids often interfere with the analysis [3].

In this paper we describe a fast accurate method to determine α -tocopherol in plasma by high-performance liquid chromatography. To our knowledge this technique has not been used for plasma samples. The method has been applied to plasma from healthy individuals as well as to plasma from pre-term newborn infants. Detection of vitamin E deficiency in the plasma of such infants may explain anaemia [4]. The results of this latter study will be reported elsewhere.

EXPERIMENTAL

Materials

n-Hexane (analytical grade, redistilled once before use), diisopropyl ether and isopropanol (spectroscopic grade) were purchased from BDH (Poole, Great Britain). α -Tocopherol was obtained from Merck (Darmstadt, G.F.R.) and the mixture of β -, γ - and δ -tocopherol was a generous gift from AB Ferrosan (Malmö, Sweden). α -Tocopheryl acetate was of purum quality from Fluka (Buchs, Switzerland).

Apparatus

A Waters Model ALC/GPC 204 liquid chromatograph equipped with a U6K loop injector and a Model 440 UV spectrophotometer was used. The outlet of the injector was connected to a stainless-steel column (60 cm $\times$ 2 mm I.D.) packed with Corasil I (purchased from Waters Assoc., Milford, Mass., U.S.A.). The column was eluted with *n*-hexane—diisopropylether (96:4) at a flow rate of 60 ml/h. The eluent was de-gassed by ultrasonication for 15 min before use. The absorbance at 280 nm was monitored at a chart speed of 0.5 cm/min. Peak areas were automatically obtained by means of a Varian CDS 111 chromatography data system.

RESULTS AND DISCUSSION

Preparation of standard solutions

Eight standard mixtures of α -tocopherol and α -tocopheryl acetate in *n*-hexane were prepared and analyzed by high-performance liquid chromatography. The solutions were stored under nitrogen in a refrigerator when not in use. The plot of peak area ratios *vs.* concentration ratios for the components in the calibration mixtures shows a good linearity (correlation coefficient, $r = 0.9986$) for α -tocopherol concentrations in the range 6–60 μ mole/l.

Preparation of samples

Human blood collected in EDTA tubes was immediately centrifuged and the plasma fraction was pipetted off. In a typical experiment 500 μ l 99.5% ethanol, containing 26.6 μ mole/l α -tocopheryl acetate, were added to 500 μ l plasma. Five-ml conical centrifuge tubes were used. After the addition of 500 μ l *n*-hexane the tube was stoppered and the sample was carefully cyclo-mixed. After centrifugation for 10 min at 30000 *g*, 15 μ l of the organic layer was directly injected into the column. The smallest volume of plasma that was extracted, was 100 μ l.

Chromatogram

As shown in Fig. 1, α -tocopherol (d) and the internal reference compound (c) are well separated (retention times 5.1 min and 3.3 min, respectively) but there are other peaks in the chromatogram that are not completely resolved. To identify some of these peaks, vitamin K₁, vitamin A, β -carotene and the β -, γ - and δ -tocopherols were injected separately and as a mixture. These compounds were also added one by one to a sample. The compounds which are extractable with *n*-hexane are indicated in the chromatogram (a–e). Unfortunately, γ -tocopherol, which has been determined from plasma samples (after silylation of the phenol group) by gas chromatography [5], could not be completely separated from other compounds in our system. Perhaps the use of a fluorescence detector instead of a UV detector, as used by Van Nierkerk [6] and by Abe and co-workers [7,8] in their studies on free tocopherols in plant extracts, would have improved the method. To make sure that no peaks in our system were hidden under the α -tocopherol peak, the sample was recycled several times. No separation into further peaks was observed.

Another mobile system was also tested in which isopropanol (0.15%) was

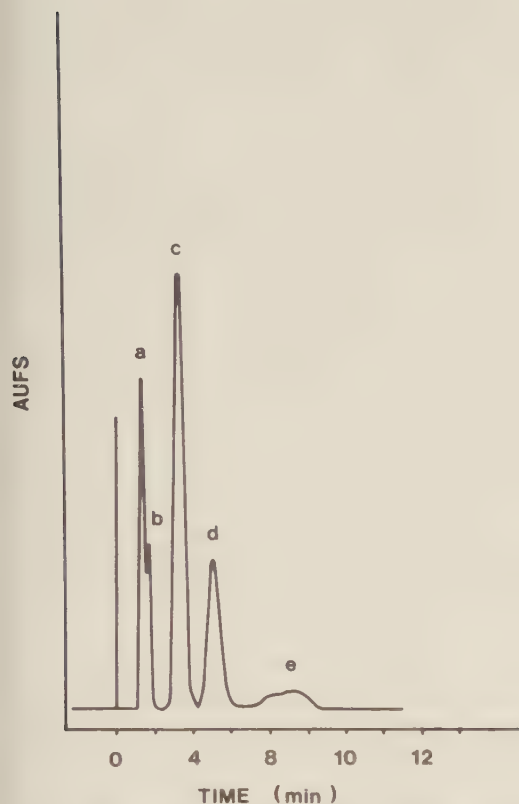


Fig.1. High-performance liquid chromatogram of α -tocopherol (d) in serum Peaks: a, β -carotene; b, vitamin K; c, α -tocopheryl acetate. The broad peak, e, was found to be due to both β - and γ -tocopherol and some other unidentified compounds. Experimental conditions are given in the text.

exchanged for diisopropyl ether (4%). The results of the α -tocopherol determination in this system compared to the one used above agreed within 2.4%. The concentration of α -tocopherol in plasma from fourteen healthy individuals varied between 17.0 and 39.9 $\mu\text{mole/l}$. This range is in good agreement with that found in the gas chromatographic study [5].

The precision and reproducibility of the method was tested by injection of the same plasma sample three times a day over a period of three days. Small variations in retention times were noticed but this did not disturb the analysis. The mean value of α -tocopherol was found to be 39.9 $\mu\text{mole/l}$, with an S.D. of 2.31 $\mu\text{mole/l}$, giving a coefficient of variation of 6.0%.

The lower limit of detection of a sample (5 μl injected) was about 6 $\mu\text{mole/l}$. The limit could be lowered if α -tocopherol was added to the sample in order to reach the linear range of the method. The recovery of added α -tocopherol to the sample was 100%. The minimum detectable amount of pure α -tocopherol was found to be 8.4 pmole, which value corresponds to twice the noise level.

The method described here is both fast and easy to apply and is suitable for small sample volumes (100 μl). It can be recommended as a complement

to the widely used spectrophotometric method [3] in which compounds such as the carotenoids often interfere with the analysis.

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CHROMBIO. 458

Note

Quantitation of serum tocopherols by high-performance liquid chromatography with fluorescence detection

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d- α -Tocopherol is considered to have the highest biological activity of the naturally occurring forms of vitamin E [1]. Therefore, vitamin E status in human populations is usually assessed in terms of the α -tocopherol level in blood. The other tocopherols (β -, γ - and δ -tocopherol), however, are an important part of the daily vitamin E intake [2]. The significance of the non- α -tocopherols in human nutrition is not known. This depends to some extent on the lack of simple and rapid methods for their determination in serum samples. The chromatographic methods hitherto used to quantify the various tocopherols in serum are based on either gas chromatography (GC) [3] or thin-layer chromatography (TLC) [4]. Our previously described high-performance liquid chromatography (HPLC) method with UV detection [5] was inadequate for resolving the various tocopherols in serum from other endogenous compounds, but in a recent paper [6] it is indicated that HPLC combined with fluorescence detection can be used to separate the tocopherols in vegetable oils.

This paper describes a refined method, using *dl*-tocol as an internal standard, to quantify the various tocopherols in serum samples. The method has been applied to serum samples from healthy individuals and from mothers and their infants (cord blood).

EXPERIMENTAL

Reagents and chemicals

n-Hexane (analytical-reagent grade), redistilled once before use, was purchased from Rathburn Chemicals, Walkerburn, Great Britain. Diisopropyl

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ether and isopropanol (spectroscopic grade) were purchased from BDH, Poole, Great Britain. α -Tocopherol was obtained from Merck, Darmstadt, G.F.R. β -, γ - and δ -tocopherol was a gift from Dr. K. Abe, Eisai Research Labs., Bunkyo-ku, Tokyo, Japan. *dl*-Tocol was obtained from Koch-Light, Colnbrook, Great Britain.

Instrumentation

Normal-phase HPLC was performed utilizing a Waters Model ALC/GPC 204 liquid chromatograph equipped with a U6K loop injector and a μ Porasil column (10 μ m particle size; Waters Assoc., Milford, Mass., U.S.A.). The column was eluted with *n*-hexane-diisopropyl ether (92:8) at a flow-rate of 2.5 ml/min. The fluorescence intensity of the column eluent was monitored continuously using a Schoeffel variable-wavelength spectrofluorimeter (Schoeffel Instrument Corp., Westwood, N.J., U.S.A.) equipped with a deuterium lamp. The attenuation was in the range 0.1–0.2. The excitation wavelength was set at 295 nm. The instrument was equipped with a cut-off emission filter (370 nm). For chromatographic comparison we also recorded the absorbance of the eluent at 280 nm with a Waters Model 440 UV detector.

Mass spectra were obtained on a JEOL JMS D-300 instrument equipped with a combined electron impact–chemical ionization ion source and a direct inlet probe. The mass spectrometer was coupled to a JMA 2000 mass data analysis system.

Preparation of standard solutions

Four standard mixtures with increasing concentrations of α -, β -, γ - and δ -tocopherol and a constant concentration of the *dl*-tocol internal standard (21.5 μ mol/l) were prepared and analysed by HPLC. Peak areas were determined by triangulation (peak base width $\times$ peak height). The fluorescence peak-area ratios of the standard α -, β -, γ - or δ -tocopherol and the internal reference compound were plotted against the corresponding concentration ratios. The graphs showed good linearity in the following concentration ranges: α -tocopherol, 5–46 μ mol/l; β -tocopherol, 0.5–3 μ mol/l; γ -tocopherol, 1–10 μ mol/l; δ -tocopherol, 0.1–1 μ mol/l. The coefficients in the equation (k = slope; l = intercept) and regression coefficients (r) are as follows: α -tocopherol, k = 0.887, l = 0.024, r = 1.000; β -tocopherol, k = 1.001, l = 0.039, r = 0.999; γ -tocopherol, k = 0.983, l = 0.017, r = 1.000; and δ -tocopherol, k = 1.142, l = 0.060, r = 0.999.

Preparation of samples

Blood samples were obtained by venipuncture from ten healthy individuals (five males and five females), from fifteen mothers immediately after delivery and from the cord blood of their infants. The serum was removed, frozen and stored at -20° until taken for analysis. To each serum sample (aliquots of 500 μ l) were added 500 μ l of 99.5% ethanol containing 10.75 nmol of *dl*-tocol. After the addition of 1 ml of *n*-hexane and Vortex-mixing for 30 sec the samples were centrifuged for 5 min at 30,000 *g*. The organic layer was removed by pipette and evaporated to dryness in a stream of nitrogen. When completely dry the residue was redissolved in 50 μ l of *n*-hexane. A 10–20- μ l volume of

the extract was injected into the column. The same procedure was also applied to smaller serum samples (down to 100 μ l).

RESULTS AND DISCUSSION

Chromatogram

Fig. 1a shows a typical chromatogram of an extract from a serum sample with *dl*-tocol as internal standard, in which α -, β - and γ -tocopherol can easily be identified. Their relative retention times are 0.37, 0.51 and 0.59, respectively. δ -Tocopherol is not observed. It is important to note that the true retention time varied by at most 5% from day to day.

A chromatogram of an extract from the serum of an infant given a lipid emulsion intravenously (Intalipid; Vitrum, Stockholm, Sweden) is shown in Fig. 1b. Intralipid is derived from fractionated soy-bean oil, which is rich in γ - and δ -tocopherol. In this chromatogram γ -tocopherol is the predominant vitamin E form. The presence of δ -tocopherol in the chromatogram of this serum extract is of interest.

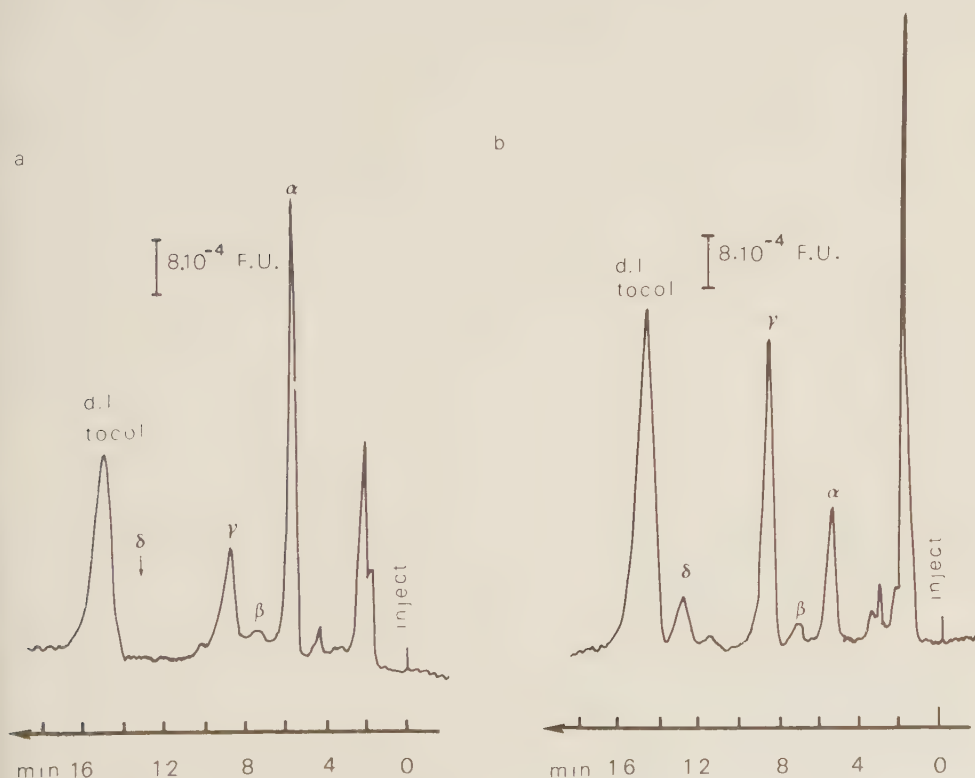


Fig. 1. HPLC separation of tocopherols in serum. (a) Normal serum; (b) serum from an infant given Intralipid. Conditions: column, μ Porasil; eluent, *n*-hexane-diisopropyl ether (92:8); flow-rate, 2.5 ml/min (1000 p.s.i.); temperature, ambient; fluorescence detection; internal standard, *dl*-tocol.

Precision, sensitivity and selectivity

The recoveries of added α -, β -, γ - and δ -tocopherol to a serum sample were found to be 92% for δ -tocopherol and 100% for the others. The precision and reproducibility of the method were tested by analysing extracts from two different serum pools twice a day over a period of 5 days. The coefficients of variation (mean for the two serum samples) were determined to be 1.5%, 22% and 7% for α -, β - and γ -tocopherol, respectively. The high coefficient of variation for β -tocopherol is probably due to the small amount present in serum. The minimal detectable amount of injected pure α -tocopherol was 21 pmol, which corresponds to twice the noise level. The detectable amount varied only slightly for the other tocopherols, as the responses were almost identical at a given concentration.

Another chromatographic system was also tested in order to ensure that no peaks in the system described above are hidden under the tocopherol peaks. The column was exchanged for a more polar column (μ -NH₂-Bondapak, 5 μ m particle size; Waters Assoc.) which was eluted with *n*-hexane—isopropanol (98:2). With this system we obtained a good separation of α - and β -tocopherol in a serum extract, but γ -tocopherol was separated less well from a new peak not observed in the other system (see Fig. 2). However, an estimation of

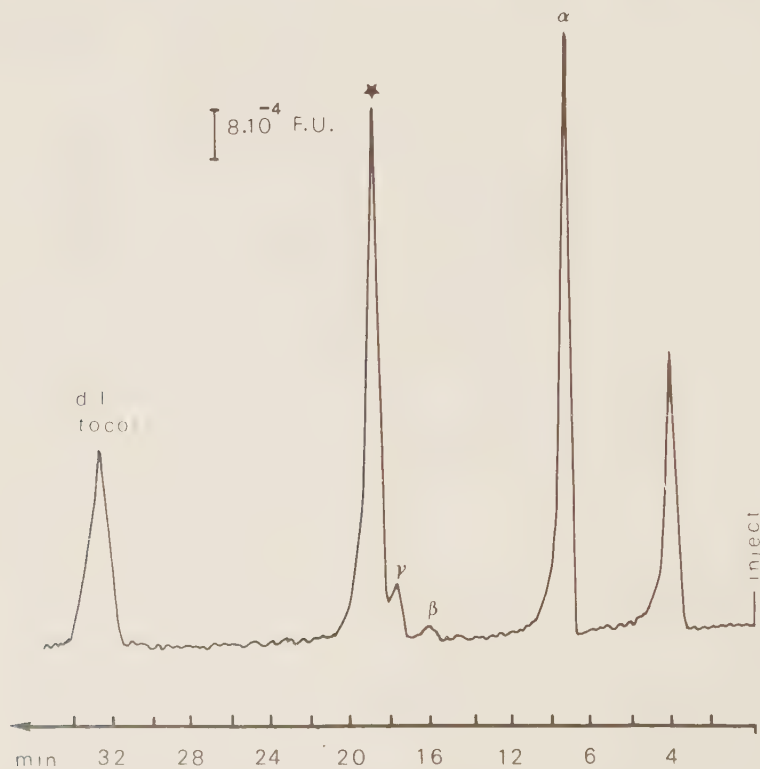


Fig. 2. HPLC separation of tocopherols in normal serum. Conditions: column, μ -NH₂-Bondapak; eluent, *n*-hexane—isopropanol (98:2); flow-rate, 1.5 ml/min (800 p.s.i.); temperature, ambient; fluorescence detection; internal standard, *dl*-tocol. The peak marked with an asterisk is not identified.

the γ -tocopherol content in a sample agreed within 10% with the result obtained on the same sample with the system described above. A fraction of the new peak in this chromatographic systems was collected and analysed by electron-impact and chemical-ionization mass spectrometry. A molar peak at $m/e = 368$ was found. The structure of the compound was not investigated further.

A quantitative evaluation of the α -tocopherol content in serum samples using UV detection showed agreement to within 3% with fluorescence detection.

Tocopherols in serum

Concentrations of the tocopherols in different serum extracts are presented in Table I. In normal serum α -tocopherol accounted for 87% (range 82.6–93.3%), β -tocopherol for 1.2% (range 0–2.3%) and γ -tocopherol for 11.8% (range 6.7–15.7%) of the total amount of tocopherols. δ -Tocopherol could be detected in the chromatogram of a normal serum if the amount of the extract injected was increased approximately 20-fold ($<0.024 \mu\text{mol/l}$). The values found in this study are higher than those previously reported [2, 4] using TLC separation with spectrophotometric determination of the tocopherols. The reason for this is not necessarily related to the precision and selectivity of the earlier method [2], as these differences can also be attributed to different dietary conditions of the individuals investigated.

TABLE I

α -, β - AND γ -TOCOPHEROL LEVELS IN DIFFERENT SERA

Type of serum	No. of samples	Mean* ($\mu\text{mol/l}$)		
		α	β	γ
Normal	10	30.6 (16.3–48.4)	0.4 (0.0–0.7)	4.1 (1.9–6.0)
Maternal	15	37.4 (20.0–62.5)	0.2 (0.0–0.7)	5.8 (1.2–10.5)
Cord	15	6.0 (1.39–12.1)	—	0.2 (0.0–1.2)

*Range in parentheses.

Maternal and cord blood was also studied (Table I). The total tocopherol concentrations found in these sera are in good agreement with those obtained in earlier studies showing high maternal and low cord blood tocopherol values [7]. This may be attributed to differences in transport capacity, as the level of plasma β -lipoprotein (the principal plasma carrier of vitamin E) has been found to correlate well with the vitamin E level [8]. However, in previous studies the various tocopherols were not separated and determined individually. In the maternal serum investigated by us α -tocopherol accounted for 86% (range 73.5–96%), β -tocopherol for 0.5% (range 0–4%) and γ -tocopherol for 13.5% (range 4–24%). In cord blood serum the α -tocopherol concentration was approximately one sixth of the maternal serum level. β -Tocopherol could not be detected, but γ -tocopherol was observed in some of the serum extracts.

ACKNOWLEDGEMENTS

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VITAMIN E REQUIREMENTS OF PRETERM INFANTS

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ABSTRACT. Jansson, L., Holmberg, L., Nilsson, B. and Johansson, B. (Departments of Paediatrics and Clinical Chemistry, University of Lund, Malmö General Hospital, Malmö, Sweden). Vitamin E requirements of preterm infants. *Acta Paediatr Scand*, 67: 459, 1978. —Differences between feeding practices in earlier investigations prompted the present study of iron and vitamin E supplementation in breast milk fed preterm infants. A new and highly sensitive technique for quantitation of alpha-tocopherol in serum was used. Studies on 34 infants with a birth weight below 2000 g or gestational age ≤ 35 weeks showed that supplementation with 16.5 mg tocopheryl acetate/day from 10 days of age resulted in a significantly higher haemoglobin concentration and lower reticulocyte count at 8–10 weeks than supplementation with 1.5 mg/day ($p < 0.05$). Studies on 23 infants with a birth weight of 2000–2499 g revealed subnormal alpha-tocopherol levels in 2 of the infants given 1.5 mg tocopheryl acetate/day but there was no effect on the haemoglobin concentration at 8–10 weeks. There were no untoward effects of an early iron supplementation with 2–3 mg Fe^{++} (as ferrous succinate)/kg/day. It is concluded that extra supplementation with vitamin E is advisable also in breast milk fed preterm infants. A low dosage iron supplementation from 3 weeks of age is safe.

KEY WORDS: Vitamin E, breast milk, preterm

Vitamin E deficiency as a cause of haemolytic anaemia in premature infants was reported by Oski & Barness in 1967 (13). Such a relation has been confirmed by some workers (8, 11), but not by others (14, 15). This lack of unanimity may be due to several facts: The colorimetric method hitherto used for quantitative determination of vitamin E (16) has in our hands not proved reliable, especially not in the analysis of small blood volumes. Furthermore, it is difficult to compare results from different centres because of differences in feeding practices with respect to other nutrients, especially polyunsaturated fatty acids, iron, folic acid and selenium (5).

This study was designed to find the optimal dosage of vitamin E for preterm infants relative to iron intake. In contrast to most previous studies all the infants were fed breast milk until they weighed at least 2100 g. Serum alpha-tocopherol levels were monitored with a newly developed and very sensitive high pressure liquid chromatography technique (12).

PATIENTS AND METHODS

Fifty-seven infants with a birth weight of less than 2500 g were studied. All the infants were born at Malmö General Hospital between December 1975 and February 1977. The gestational age of each infant was assessed from a combination of maternal data and the external characteristics (18) and from a neurological evaluation (1). Infants with haemoglobin values below 150 g/l or above 260 g/l in the first 24 hours were not accepted. Neither were those with hyperbilirubinaemia or those who required blood transfusions. Two children with mild respiratory distress were accepted. None of the other children had any disease during the period of study.

The infants were assigned to one or the other of 2 categories according to both birth weight and gestational age: Those with a birth weight of 1000–1999 g or gestational age < 35 weeks were assigned to category A; those with a birth weight of 2000–2499 g to category B. The infants in each category were then randomly distributed among 3 groups given different therapeutic regimens:

1. Ferrous succinate (Ferromyn S, AB Hässle) (2–3 mg Fe^{++} /kg/day) from 3 weeks of age (conventional regimen).
2. Tocopheryl acetate (E-vitamin, AB ACO) (15 mg/day) from 10 days of age and ferrous succinate (2–3 mg Fe^{++} /kg/day) from 3 weeks.
3. Tocopheryl acetate (15 mg/day) from 10 days of age and ferrous succinate (2–3 mg Fe^{++} /kg/day) from 10 weeks.

The distribution of infants among the birth weight categories and therapeutic regimens is given in Table 1. The

Table 1 Distribution of the infants during the birth weight categories and the therapeutic regimens

The table shows birth weight, gestational age and haematological data at birth

Therapeutic regimen	Category A (1000–1999 g)			Category B (2000–2499 g)		
	1	2	3	1	2	3
No. of patients	13	12	9	11	8	7
Mean birth weight, g	900	1767	1866	2135	2330	2288
Mean gestational age, weeks	34.6	35	33.8	37.5	36.4	37
Mean haemoglobin, g/l	150	194	181	207	177	192
Mean reticulocyte count, $\times 10^9/l$	147	109	166	124	122	143
Mean platelet count, $\times 10^9/l$	169	151	181	213	202	217

mean birth weights, gestational ages and haematological data at birth did not differ between the groups. From 10 days of age all infants received a multivitamin preparation (Procovit, Roche), (12 ml/day) containing vitamins A, C, D, the B complex and a small amount of tocopherol acetate at 5 mg/day, and also Leucovorin (Lederle) containing 50 µg tetrahydrofolic acid/day.

The feeding routines were the following. All infants were given human pasteurized milk for the first 2 weeks or until they weighed >2100 g. They were then changed to the commercial formula Milкотал (AB Firdus) with a tocopherol content of 5 mg/l and an iron content (Ferne orthophosphate) of 12 mg/l. The children were examined haematologically within the first 24 hours and again at the age of 8–10 weeks. Blood for determination of tocopherol was drawn by venipuncture at approximately 5–6 weeks in category A and at 2–3 weeks in category B. Sera were stored at -20°C until analyzed. Haemoglobin concentration and reticulocyte and platelet counts were determined with conventional methods.

Serum alpha-tocopherol was determined by high pressure liquid chromatography (12). The equipment consisted of a Waters model ALC/GPC 204 liquid chromatograph, a UV-6 injector, a model 440 UV spectrophotometer, and a Corasil 1 straight phase column. The absorbance at 254 nm was recorded. To each serum sample (200 µl) equal volumes of 99.5% ethanol and *n*-hexane were added. The ethanol contained 20.0 µmol/l α -alpha-tocopherol acetate as an internal reference. No interfering peaks could be visualized in the spectra of serum samples not containing the internal reference. After careful cyclomixing the proteins were separated from the organic layer by centrifugation. The hexane phase was injected directly to the Corasil column.

RESULTS

In birth weight category A (1000–1999 g) the therapeutic groups were found to differ with

respect to alpha-tocopherol level as well as haemoglobin level and reticulocyte count (Table 2). Serum alpha-tocopherol was significantly lower in group 1 (low supplementary vitamin E, Fe^{++} from 3 weeks) than in group 2 or 3 ($p < 0.001$). In group 1 two infants had alpha-tocopherol levels below 11.6 µmol/l (the critical lowest level according to previous studies). These 2 infants had birth weights of 1540 and 1320 g, respectively. In groups 2 and 3 no infant showed an alpha-tocopherol concentration below 11.6 µmol/l. There was no difference between groups 2 and 3.

In group 1 the haemoglobin concentration was significantly lower, and the reticulocyte count significantly higher, than in group 2 (high dose vitamin E, Fe^{++} from 3 weeks) ($p < 0.05$). In group 3 (high vitamin E, Fe^{++} from 10 weeks) the mean haemoglobin concentration was lower than in group 2, but the difference was not statistically significant ($0.05 < p < 0.1$). The platelet count did not differ between the groups.

In birth weight category B (2000–2499 g) the serum alpha-tocopherol levels were significantly lower in group 1 than in group 2 or 3 ($p < 0.001$). In group 1 two of the infants showed serum alpha-tocopherol values below 11.6 µmol/l. The birth weights of these infants were 2280 and 2100 g, respectively. At 8–10

Table 2. *Haematological data at 8–10 weeks in preterm infants given different therapeutic regimens*Serum α -tocopherol was determined at 5–6 weeks in category A and at 2–3 weeks in category B

Therapeutic regimen ...	Category A (1 000–1 999 g)			Category B (2 000–2 499 g)		
	1	2	3	1	2	3
Haemoglobin, g/l; mean (S.D.)	97.3 (10.2) ^a	105.3 (8.4)	98.7 (5.3)	111.2 (14.2)	113.8 (6.9)	107.0 (7.5)
Reticulocytes, $\times 10^9/l$; mean (S.D.)	77.7 (36.7) ^b	49.2 (29.3)	45.8 (35.4)	27.6 (16.8)	36.6 (28.6)	38.8 (25.9)
Platelets, $\times 10^9/l$; mean (S.D.)	375 (166)	391 (185)	460 (195)	401 (165)	420 (224)	412 (213)
α -tocopherol, $\mu\text{mol/l}$; mean (S.D.)	17.8 (5.0) ^c	29.1 (5.0)	30.9 (9.9)	19.7 (7.9) ^d	26.5 (2.9)	31.3 (7.3)

^a $p < 0.05$ compared to group 2^b $p < 0.05$ compared to group 2.^c $p < 0.001$ compared to groups 2 and 3.^d $p < 0.001$ compared to groups 2 and 3.

weeks of age, however, the groups did not differ from one another in haemoglobin level, reticulocyte or platelet count.

DISCUSSION

Vitamin E deficiency in prematurely born infants can be attributed to several factors. The placental transfer of tocopherol is low and the tissue stores at birth are limited (3). In the most immature infants the absorption of tocopherol is impaired (11), probably because of a reduced synthesis of bile salts (19).

The function of vitamin E in the body is obscure. It is thought to have an *in vivo* anti-oxidizing effect (7), which could explain the phenomena observed in premature infants. Melhorn & Gross (10) found the haemoglobin to be decreased after administration of large doses of medicinal iron. Williams et al. (20) found a deleterious effect on haemoglobin values when they fed premature infants a formula high in linoleic acid and iron. A large amount of iron would increase the oxidant stress on the erythrocyte membrane. Furthermore, a large amount of polyunsaturated fatty

acids in the diet would increase erythrocyte linoleic acid (6) and thus the amount of oxidizable substrate. This would result in an increased fragmentation of the erythrocyte membrane and even a normal level of α -tocopherol would sometimes not be sufficient to prevent red cell destruction under such conditions.

A second major biological antioxidant is glutathione peroxidase, recently identified as a selenium-dependent enzyme (7). Cow's milk is poor in selenium as it contains only half the amount of selenium in breast milk (9). Preterm infants fed a cow's milk formula for more than 60 days show a marked decline in plasma selenium levels as well as in erythrocyte glutathione peroxidase activity (5). The relation between this relative selenium deficiency and haemolytic anaemia of the premature infant is still not clear. However, with reference to the above observations the ideal formula should be high in selenium and tocopherol, low in linoleic acid and iron. Human milk should meet these requirements better than any commercial formula.

In contrast to most previous studies the in-

infants in our series received breast milk until they had reached such a degree of maturity that the risk of developing tocopherol-dependent haemolysis was low. Our patients then probably got an adequate amount of polyunsaturated fatty acids and selenium. To eliminate other possible deficiencies all infants received a multivitamin preparation and tetrahydrofolic acid. Thus the effects of tocopherol and iron administration could be studied in the physiological setting.

The present study shows that also under optimal feeding conditions, tocopherol substitution is warranted for preterm infants with a birth weight of less than 2000 g. The amount of tocopherol in common multivitamin preparations is not sufficient, as our group 1, who got only such a preparation (supplying tocopherol in a dose of 1.5 mg/day) had a significantly reduced serum tocopherol content and signs of hyperhaemolysis with a lower haemoglobin concentration and a higher reticulocyte count. The daily dose of 16.5 mg tocopherol given to groups 2 and 3 is in the range suggested by Dallman (3), and seems to be quite sufficient, as none of the infants in this group had critically low alpha-tocopherol levels according to previous experiences. Even in the smallest infants a satisfactory level of alpha-tocopherol was reached indicating a resorption good enough under the conditions of feeding used. However, our material contained only few infants with a gestational age below 32 weeks. No untoward effects of the doses of vitamin E were seen.

Small preterm infants run a risk of developing iron deficient anaemia unless the food is supplemented with iron. However, early use of large doses of ferrous sulfate should be avoided because it increases the tendency to haemolysis (14). A common practice in Sweden is to start iron supplementation to preterm infants at 3 weeks of age (dose 2–3 mg/kg Fe^{++} as ferrous succinate).

In the present study there was no statistically significant difference in haemoglobin concentration or reticulocyte count between

groups 2 and 3, e.g. the infants who received vitamin E and iron, and those given only vitamin E. Furthermore, iron seemed not to influence vitamin E absorption, as the alpha-tocopherol levels proved equal in groups 2 and 3.

As the mean concentration of haemoglobin was highest in group 2 it could be concluded that iron in the doses used at least caused no harm to red cell survival. If iron supplementation is delayed there may be a risk of iron deficiency. Thus, judging from the present study there is no reason to modify the conventional iron regimen.

Our findings warrant the following conclusions: Breast milk fed premature infants with a gestational age of less than 35 weeks or a birth weight below 2000 g seem to benefit from supplementary vitamin E (15 mg tocopheryl acetate/day) given from 10 days of age for a period of 8–10 weeks. Ferrous succinate (2–3 mg Fe^{++} kg/day) should be given from 3 weeks of age.

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Vitamin E and fatty acid composition of human milk^{1, 2}

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ABSTRACT The vitamin E and fatty acid composition of human milk was determined in 40 milk samples (six colostral, 10 transitional, and 24 mature) obtained at different stages of lactation. Vitamin E was determined by high performance liquid chromatography with fluorescence detection of the various tocopherols. The total tocopherol level was significantly higher in early milk than in mature milk. The difference was due to a high content of α -tocopherol, as the content of β - and γ -tocopherol was similar in the three milk types. The total tocopherol content in mature milk correlated significantly with both the total lipid and the linoleic acid content. Significantly higher tocopherol/linoleic acid ratios were found in both colostrum and transitional milk than in mature milk. The colostral milk differed from the other milk types in fatty acid composition, as it had a lower content of lauric acid and a higher content of arachidonic acid and docosahexaenoic acid. The linoleic acid levels reported here are considerably higher than those reported previously in Sweden. Still, the ratio of α -tocopherol equivalent/linoleic acid exceeded 0.5 mg/g in all but three milk samples. *Am. J. Clin. Nutr.* 34: 8–13, 1981.

KEY WORDS Human milk, lactation, infant nutrition, vitamin E, tocopherol, fatty acids

Introduction

Fat constitutes about 50% of the total caloric value of human milk (1). The composition of the milk lipids varies to some extent with the mothers' diet, an increase in dietary polyunsaturated fat for example increasing the polyunsaturated fatty acid (PUFA) content of milk (2–3). Guthrie et al. (4) recently showed that in American mothers the breast milk seems to contain more PUFA today than it did 20 years ago. This change coincides with an increased consumption of vegetable oils, rich in polyunsaturated lipids. A high concentration of PUFA in human milk is not necessarily advantageous for the infant. In preterm infants a high intake of PUFA can lead to hemolysis, because the infants have a relative deficiency of vitamin E (5).

Previous studies of the vitamin E and fatty acid composition of human milk have been inadequate. Thus the various tocopherols in human milk have not been determined separately, and properly studied regarding their quantitative variation with that of the various fatty acids. Vitamin E consists of eight naturally occurring substances (α -, β -, γ -, δ -tocopherol and the corresponding tocotrienols). It appears that the vitamin E con-

tent of human milk has hitherto been assessed with colorimetric methods, either as total tocopherols (6) or α -tocopherol after separation by thin layer chromatography (7). Actually, γ -tocopherol nowadays constitutes an important part of the vitamin E intake, because of the increasing consumption of vegetable oils (8).

This study concerns the level of α -, β -, and γ -tocopherol in human milk at different stages of lactation and their relation to the total lipid content and the fatty acid composition.

Materials and methods

Thirty-four samples of human milk were collected from 34 healthy voluntary donors to the human milk bank in Malmö General Hospital. The milk was collected at home by the mother, expressed by breast pump after the regular feed to the baby, during a period of 2 days, and stored at home in the refrigerator until delivered to the milk bank. There the milk was immediately homogenized by vigorous shaking. Two 5 ml aliquots were

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removed, immediately frozen and stored at -20°C until analyzed. Of the 34 milk samples 10 were collected on days 6 to 10 postpartum (transitional milk), and 24 from the 12th day to 5th month (mature milk). Six colostrum samples were collected from six healthy mothers on the 4th day postpartum. These samples were expressed by breast pump before the first morning feed, immediately frozen, and stored at -20°C until analysed.

Vitamin E determination

The various tocopherols in human milk were determined with high performance liquid chromatography with fluorescence detection (9). This method which has been developed for serum samples, was slightly modified with respect to the extraction procedure, and proved suitable also for milk samples. The equipment consisted of Waters Model ALC/GPC 204 liquid chromatograph, a U6K injector, a Waters 30 cm μ -Porasil column (particle size 10 μ ; column diameter 4 mm; Waters Associates, Milford, MA) and a Schoeffel variable wavelength spectrofluorimeter equipped with a deuterium lamp. The excitation wavelength was 295 nm and a cutoff emission filter of 370 nm was used.

To each milk sample (500 μl) was added an equal volume of 99.5% ethanol, containing 21.5 $\mu\text{mol/l}$ of the internal standard *dl*-tocol. After addition of 1500 μl *n*-hexane, sample was cyclomixed for 30 s and centrifuged at ambient temperature for 5 min at $30,000 \times g$. The organic layer was removed by pipette, and evaporated to dryness in a stream of nitrogen. The residue was redissolved in 50 μl *n*-hexane. Twenty microliters of the extract was injected into the column. The various tocopherols were well separated despite the large amount of lipids in the injected extract, and the recovery of added tocopherols was similar to that obtained on analysis of serum extracts (100% for α -, β -, and γ -tocopherol; 92% for δ -tocopherol). The column was washed with methanol after every 10 samples.

α -, β -, and γ -tocopherol values have been expressed in $\mu\text{mol/l}$. The total tocopherols have also been converted to α -tocopherol equivalent (mg/dl) (10). According to Bieri and Evarts (11), the vitamin E activity of γ -tocopherol relative to α -tocopherol was estimated to 10%.

Total lipid and fatty acid determination

Lipids were extracted from 2 ml milk with 6 vol chloroform:methanol (1:1 vol/vol) for 1 h at room temperature. The precipitate obtained after centrifugation was rinsed once with 5 ml chloroform:methanol (1:1). Eight milliliters of NaCl (10 mg/ml) was added to the

combined extracts and the lower phase was washed once with 10 ml methanol:water (1:1 v/v). The chloroform phase was taken to dryness and total lipid was quantitated gravimetrically. The coefficient of variation for the determination of total lipid was 5.8%, calculated from duplicate extractions of six samples. The residue was dissolved in toluene and an aliquot was transesterified (12). Fatty acid methyl esters were analysed by gas chromatography as described previously (12), using EGSS-Y as the stationary phase (Applied Science, State College, PA). Data are expressed as weight %. Unsaturated acids were identified by comparison of the retention times of the following methyl esters: 18:1 (n-9), 18:2 (n-6), 18:3 (n-3), 20:1 (n-9), 20:2 (n-6), 20:4 (n-6), 22:6 (n-3). Eicosatrienoic acid (20:3) was tentatively identified, but it was observed that the n-9 isomer was not included in this peak. The contribution of different fatty acid isomers was not studied. Some minor peaks observed were not included in the calculations.

Statistics

The differences between the groups were calculated with student's *t* test.

Results

Vitamin E

The total tocopherol contents as well as the content of α -, β -, and γ -tocopherol in 40 human milk samples are given in Table 1. The means $\pm$ SD of total tocopherol in the different types of milk were: colostrum 26.1 ± 13.7 $\mu\text{mol/l}$; transitional milk 13.5 ± 4.7 $\mu\text{mol/l}$; and mature milk 9.4 ± 5.1 $\mu\text{mol/l}$. The difference between early milk (i.e., colostrum and transitional milk combined) and mature milk was statistically significant ($p < 0.005$). The same differences were found when the total tocopherol values were converted to mg α -tocopherol equivalent (Table 1).

The means $\pm$ SD of the concentrations of α -tocopherol were: colostrum 23.0 ± 12.6 $\mu\text{mol/l}$; transitional milk 10.4 ± 4.2 $\mu\text{mol/l}$; and mature milk 7.2 ± 3.9 $\mu\text{mol/l}$. The difference between colostrum and transitional

TABLE 1
 α -, β -, γ -, and total tocopherol levels ($\mu\text{mol/l}$) and α -tocopherol equivalent levels (mg/dl) in human milk*

Type of milk	No. of samples	α -	β -	γ -	total tocopherol	α -tocopherol equivalent
Colostrum	6	$23.0 \pm 12.6^{\dagger}$	0.2 ± 0.02	3.9 ± 1.0	26.1 ± 13.7	1.00 ± 0.55
Transitional	10	10.4 ± 4.2	0.2 ± 0.1	2.4 ± 1.4	13.5 ± 4.7	0.48 ± 0.18
Mature	24	$7.2 \pm 3.9^{\ddagger}$	0.2 ± 0.1	2.1 ± 1.3	$9.4 \pm 5.1^{\S}$	$0.32 \pm 0.18^{\S}$

* Means $\pm$ SD.

$^{\dagger} p < 0.02$ compared to transitional.

$^{\ddagger} p < 0.005$ compared to transitional.

$^{\S} p < 0.005$ compared to combined colostrum and transitional.

milk was statistically significant ($p < 0.02$), as was that between transitional milk and mature milk ($p < 0.005$). There were no significant differences in absolute β - and γ -tocopherol levels between the three milk categories.

The relative levels of α - and γ -tocopherol were calculated as percentages of the total tocopherols. The means (and ranges) of these percentage values were for α -tocopherol: colostrum 87.3% (83.3 to 90.7); transitional milk 79.7 (58.5 to 91.6); and mature milk 76.4 (64.7 to 94.1). The difference was statistically significant ($p < 0.05$) when early milk, i.e., colostrum + transitional milk, was compared with mature milk. The means (and ranges) of the relative level of γ -tocopherol showed the opposite pattern, viz. colostrum 11.5% (8.1 to 16.7), transitional milk 18.2% (7.0 to 30.0), and mature milk 21.5% (5.0 to 32.4). The difference between colostrum + transitional milk and mature milk was statistically significant ($p < 0.05$).

The effects of freezing and heating on tocopherol were studied. Five unfrozen milk samples were analyzed before and after storage at -20°C for 4 wk and after pasteurization ($+70^{\circ}\text{C}$ for 5 min). These procedures caused no significant loss of tocopherol (less than 5%).

Total lipids and fatty acids

The total lipid and fatty acid concentration in the 40 samples are given in Table 2. The mean total lipid content $\pm$ SD was lower in colostrum milk 14.9 ± 3.1 g/l, than in transitional 30.0 ± 19.3 g/l or in mature milk 33.0 ± 10.6 g/l ($p < 0.005$). Colostrum and transitional milk differed also in fatty acid composition. Significantly lower colostrum levels were found for C 12:0 ($p < 0.025$). Higher colostrum levels were found for C 16:1 ($p < 0.005$), C 18:1 ($p < 0.005$), C 20:1 ($p < 0.001$), C 20:2 ($p < 0.005$), C 20:4 ($p < 0.02$), and for C 22:6 ($p < 0.001$). The transitional milk differed from mature milk only in content of C 22:6, which was higher in the former ($p < 0.005$).

Tocopherol/total lipid ratio

The total tocopherol content of mature milk varied with the total lipid content ($r = 0.52$, $p < 0.01$). No such correlation was

found in the early milk samples (colostrum and transitional). The tocopherol/lipid ratios (Table 3) were calculated from the total tocopherol and total lipid values (Tables 1 and 2). The means $\pm$ SD expressed in μmol tocopherol/g lipid were: colostrum 1.84 ± 1.1 ; transitional milk 0.46 ± 0.16 ; and mature milk 0.29 ± 0.11 . The difference between colostrum and transitional milk was significant ($p < 0.005$), as was that between transitional and mature milk ($p < 0.001$). The tocopherol/lipid ratios were also expressed in mg α -tocopherol equivalent/g lipid (Table 3).

Tocopherol/linoleic acid ratio

The total tocopherol content of mature milk varied closely with the linoleic acid content ($r = 0.68$, $p < 0.001$) (Fig. 1). The α -tocopherol content showed a similar correlation with linoleic acid ($r = 0.70$, $p < 0.001$), but not the β - or γ -tocopherol content. The linoleic acid content expressed as a percentage of the total fatty acid content, was not significantly correlated to the content of different tocopherols or to total lipids. The means $\pm$ SD tocopherol/linoleic acid ratios expressed in $\mu\text{mol}/\text{mmol}$ (Table 3) were: colostrum 4.64 ± 2.77 , transitional milk 1.12 ± 0.45 , and mature milk 0.65 ± 0.23 . These

TABLE 2
Total lipid content (g/l) and fatty acid composition (weight %) in 40 human milks*

Type of milk	Colostrum (n = 6)	Transitional (n = 10)	Mature (n = 24)
Total lipid g/l	$14.9 \pm 3.1^{\dagger}$	30.0 ± 19.3	33.0 ± 10.6
Fatty acids %			
12:0	$2.6 \pm 0.8^{\ddagger}$	4.5 ± 1.6	4.4 ± 1.5
14:0	5.6 ± 0.9	6.5 ± 1.9	6.3 ± 1.6
16:0	23.4 ± 1.2	23.5 ± 1.4	23.0 ± 1.9
16:1	$4.3 \pm 0.5^{\S}$	3.7 ± 0.5	3.8 ± 0.8
18:0	7.1 ± 1.2	8.2 ± 0.9	8.4 ± 1.0
18:1 (n-9)	$39.5 \pm 1.4^{\S}$	37.1 ± 1.4	38.0 ± 2.7
18:2 (n-6)	12.1 ± 1.0	12.8 ± 2.8	12.9 ± 2.2
18:3 (n-3) + 20:0	1.2 ± 0.2	1.3 ± 0.4	1.4 ± 0.3
20:1 (n-9)	1.3 ± 0.2	0.7 ± 0.2	0.6 ± 0.2
20:2 (n-6)	$0.6 \pm 0.1^{\S}$	0.4 ± 0.1	0.3 ± 0.1
20:3	0.5 ± 0.2	0.3 ± 0.1	0.3 ± 0.1
20:4 (n-6)	$0.8 \pm 0.2^{\ddagger}$	0.5 ± 0.2	0.4 ± 0.1
22:6 (n-3)	1.0 ± 0.2	0.5 ± 0.2	0.3 ± 0.1

* Means $\pm$ SD.

$^{\dagger} p < 0.005$ compared to transitional and mature.

$^{\ddagger} p < 0.025$ compared to transitional and mature.

$^{\S} p < 0.005$ compared to transitional and mature.

$| p < 0.001$ compared to transitional and mature.

TABLE 3

The ratios of total tocopherol/total lipid, α -tocopherol equivalent/total lipid, total tocopherol/linoleic acid, and α -tocopherol equivalent/linoleic acid in human milk*

Type of milk	Colostrals (n = 6)	Transitional (n = 10)	Mature (n = 24)
Total tocopherol/total lipid ($\mu\text{mol/g}$)	$1.84 \pm 1.1^\dagger$	0.46 ± 0.16	$0.29 \pm 0.11^\ddagger$
α -Tocopherol equivalent/total lipid (mg/g)	$0.54 \pm 0.35^\dagger$	0.17 ± 0.06	$0.10 \pm 0.04^\ddagger$
Total tocopherol/linoleic acid ($\mu\text{mol}/\text{mmol}$)	$4.54 \pm 2.77^\dagger$	1.12 ± 0.45	$0.65 \pm 0.23^\ddagger$
α -Tocopherol equivalent/linoleic acid mg/g	$6.23 \pm 3.90^\dagger$	1.43 ± 0.66	$0.78 \pm 0.28^\ddagger$

* Means $\pm$ SD.

$^\dagger p < 0.005$ compared to transitional.

$^\ddagger p < 0.001$ compared to transitional.

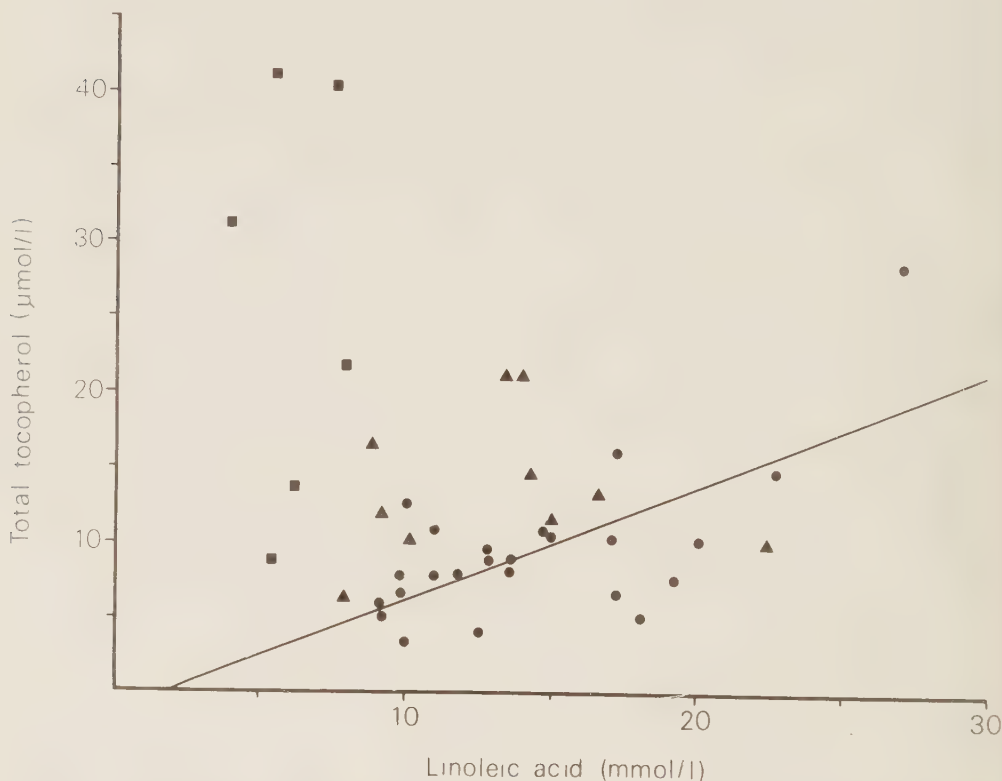


FIG. 1. The relationship of total tocopherol content ($\mu\text{mol/l}$) and linoleic acid content (mmol/l) in 40 samples of human milk at different stages of lactation. ■: six colostral, ▲: 10 transitional, and ●: 24 mature milk samples. The regression line for the mature milk samples is drawn.

differences were significant both when colostrum was compared with transitional milk ($p < 0.005$), and transitional was compared with mature milk ($p < 0.001$). The same differences were found when the α -tocopherol equivalent/linoleic ratios were calculated (Table 3).

Discussion

Vitamin E

In the present investigation the total tocopherol levels in human milk were higher than in most previous studies (13–15), possibly with the exception of Millar and Shep-

pard (16). None of the earlier investigators has, however, studied the vitamin E content of milk at different stages of lactation. We found a marked variation of the tocopherol content of milk samples obtained on different stages of lactation. Thus colostrum contained about 3 times as much tocopherol as mature milk.

A variation in total tocopherol content during lactation and similar to that found by us in human milk has been observed earlier in bovine milk. Herting and Drury (7) found vitamin E levels in bovine colostrum to be about seven times those in mature milk, although the two types of milk were similar in average lipid content.

We also found a correlation between total tocopherol and lipid content of mature milk which is in accordance with the finding by Harris et al. (13). No correlation was found between tocopherol and lipid in colostrum and transitional milk samples. A comparison between the tocopherol/lipid ratios in colostrum and transitional milk indicated rather that the high colostrum tocopherol level is not secondary to an increased lipid secretion. This is in accord with the findings for bovine milk (7) and can probably be explained by the high concentration of β -lipoprotein in the maternal plasma (17), which suggests an increased transport capacity for vitamin E. The advantages for the infant are evident, there is a large supply of vitamin E during the first week of life, when the infant's tissues stores of vitamin E are low and need supplementation. Moreover, the present study shows that the high tocopherol level in the colostrum samples was due almost entirely to a high α -tocopherol content. The content of the other tocopherols identified in human milk, i.e., β - and γ -tocopherol, did not differ between milk types, but the relative content of γ -tocopherol was highest in mature milk. The nutritional significance of β - and γ -tocopherols in human milk seems, however, of little importance, since the *in vivo* activity of γ -tocopherol is probably only 10 to 15% compared to that of α -tocopherol (18).

Total lipids and fatty acids

The mean total lipid levels found in transitional milk and mature milk are in good agreement with earlier studies (19), but the

colostral levels seem lower than those previously reported. Macy et al. (20) found an average lipid content of 29 g/l in pooled 24 h samples of colostrum. The lipid content in human milk increases during the feeding (19), and therefore our values may not represent true averages for the total feeding. The fatty acid composition in human milk, seems, however, to be almost constant during the feeding (21).

Like the lipid content the fatty acid composition was practically the same in transitional as in mature milk. The colostrum milk differed from the other types of milk in several respects. The most striking difference was the lower proportion of lauric acid (C 12:0) and the higher levels of the long chain polyunsaturated fatty acids especially arachidonic acid (C 20:4) and docosahexaenoic acid (C 22:6). The physiological significance of these differences in fatty acid composition in human milk has been discussed by Crawford et al. (22), who believed that the long chain polyunsaturated fatty acids were important for the braincell growth of the infant. Arachidonic acid has also received wide attention because it is a precursor of the prostaglandins. The most abundant essential fatty acid in human milk was linoleic acid (C 18:2). In the present study we found that linoleic acid accounted for about 13% of the total fatty acids. Such values are substantially higher than those reported by Söderhjelm in 1953 (2) but agree more with those reported in a recent study from the United States by Guthrie et al. (4) who found higher linoleic levels in human milk today than 20 yr ago. Our findings show the same tendency in Sweden and the increase is probably attributable to the increased consumption of margarines rich in linoleic acid by the mothers.

Vitamin E/linoleic acid ratio

The nutritional need of vitamin E must, however, be evaluated on a relative basis, because excess in dietary PUFA can produce excessive peroxidation and thus increase the vitamin E requirement (5, 23, 24). According to American recommendation (5) 0.7 IU of vitamin E should accompany each g linoleic acid in the infant formula. The present study shows both a significant correlation between the total tocopherol content and the linoleic

acid content in mature human milk (Fig. 1), and higher tocopherol/linoleic acid ratios in colostrum and transitional milk than in mature milk (Table 3). The American recommendations were fulfilled in all but three milk samples. In these the ratios were 0.40, 0.42, and 0.46 mg α -tocopherol equivalent/g linoleic acid, respectively. These three were samples of mature milk. Thus, in our material three of 40 milk samples did not satisfy the formula recommendations. The needs of the full term infant are probably satisfied anyhow. However, the preterm infant has a higher requirement of vitamin E (5 to 25 IU/day) (23, 25). Our study shows that this requirement, as a rule, is not met by human milk. 

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THE EFFECT OF DIETARY γ -TOCOPHEROL ON SERUM TOCOPHEROLS IN FORMULA-FED INFANTS

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ABSTRACT. Jansson, L., Holmberg, L. and Jakobsson, I. (Department of Paediatrics, University of Lund, Malmö General Hospital, Malmö, Sweden). The effect of dietary γ -tocopherol on serum tocopherols in formula-fed infants. *Acta Paediatr Scand*, 70:297, 1981.—The levels of α -, β - and γ -tocopherol were determined in two formulas (Nutramigen® and Soja-semp®) and in 20 infants fed either of these formulas. The γ -tocopherol concentration in Nutramigen was $22.8 \pm 1.9 \mu\text{mol/l}$; and that in Soja-semp $3.7 \pm 0.5 \mu\text{mol/l}$. This difference was reflected in the serum tocopherols of the infants, since in the 10 infants fed Nutramigen, γ -tocopherol accounted for $16.7 \pm 7.9\%$ of the total tocopherols compared to $4.2 \pm 1.8\%$ in the 10 infants fed Soja-semp. The study shows that γ -tocopherol accumulates in the serum of infants fed large amounts of it. The serum γ -tocopherol level should therefore be taken into account, when estimating the vitamin E status in humans, especially when the intake of γ -tocopherol is high.

KEY WORDS: Vitamin E, tocopherol, formula, infants

The vitamin E status in human populations has usually been appraised from the serum or plasma levels of α -tocopherols or total tocopherols. In recent years also the non- α -tocopherols (β -, γ - and δ -tocopherol) have received wide interest (1, 2, 3). This interest was roused mainly by an observed increase in the consumption of these tocopherols, especially γ -tocopherol (which is the predominant form of vitamin E in corn and soybean oil). γ -Tocopherol is thus considered a substantial part of the daily vitamin E intake. Knowledge of the effect on serum γ -tocopherol levels of an increased consumption of γ -tocopherol is still incomplete. In healthy adults, the plasma γ -tocopherol constitutes approximately 5–15% of the total tocopherols (4, 5). Horwitt et al. (6) have found γ -tocopherol to account for 30–40% of the total tocopherols in adults fed a corn oil diet for 2–4½ weeks. Such levels have, however, not been demonstrated in children. We therefore studied the serum levels of α -, β - and γ -tocopherol in two groups of in-

fants fed two formulas differing in γ -tocopherol content.

MATERIAL AND METHODS

After parental consent venous blood samples were obtained from 20 infants before the early morning feed. All the infants had been on a cow milk protein free diet for at least two months because of suspected intolerance to cow's milk protein. Ten infants had received a diet based on Nutramigen® formula (Mead Johnson Corp. USA), and 10 a diet based on Soja-semp® formula (AB Semper Sweden). According to the manufacturer's descriptions vitamin E contents in the formulas are for Nutramigen 10.6 mg/l (24.6 $\mu\text{mol/l}$) (dl- α -tocopherol) and for Soja-semp, 8 mg/l (total tocopherols). All the infants had a normal weight for age when examined (>-2 S.D. and $<+2$ S.D.). The mean (range) age at the examination was for the Nutramigen group 8.9 (4–15) months, and for the Soja-semp group 6.8 (3–15) months. Sera were immediately separated and stored at -20° until analysed.

The different tocopherols in the serum samples and in the formulas were determined by high performance liquid chromatography with fluorescence detection as earlier described (7). Simultaneous registration of the UV absorbance at 280 nm also enabled us to identify the acetate ester of α -tocopherol (α -tocopherylacetate). Because of low sensitivity and selectivity for tocopherols with the UV-detection in this system, α -tocopherylacetate concen-

Table 1. Tocopherol content in Nutramigen and Soja-semp and in serum of infants fed these formulas

(Mean $\pm$ S.D.)

	Tocopherol concentration ($\mu\text{mol/l}$)			
	Tocopherol concentration in		Serum tocopherol concentrations	
	Nutramigen	Soja-semp	Nutramigen	Soja-semp
α	6.7 $\pm$ 1.3	15.0 $\pm$ 2.0	15.6 $\pm$ 4.9	18.4 $\pm$ 6.3
β	0.2 $\pm$ 0.2	0.2 $\pm$ 0.2	0.2 $\pm$ 0.2	0.2 $\pm$ 0.2
γ	22.8 $\pm$ 1.9	3.6 $\pm$ 0.4	3.1 $\pm$ 1.4 ^a	0.8 $\pm$ 0.5
Total		18.6 $\pm$ 2.2	18.9 $\pm$ 5.4	19.3 $\pm$ 6.5

^a $p < 0.001$ compared to Soja-semp group.

tration could not be quantitated. Samples from three different batches of formula were analysed in duplicate.

RESULTS

Formula tocopherol

The mean levels $\pm$ S.D. for α -, β - and γ -tocopherol in Nutramigen and Soja-semp are shown in Table 1. In Nutramigen also α -tocopherylacetate was identified, but not in Soja-semp.

Serum tocopherols

Table 1 shows the mean serum levels $\pm$ S.D. of α -, β - and γ -tocopherol in the two groups of

infants. No difference in total tocopherol values was found between the groups. The γ -tocopherol accounted for 16.7 $\pm$ 7.9% of the total tocopherol in the Nutramigen group and for 4.2 $\pm$ 1.8% in the Soja-semp group. The difference was statistically significant ($P < 0.001$). The range of the γ -tocopherol levels was wide (Fig. 1), but the younger infants, who were almost entirely formula fed showed the highest γ -tocopherol levels. The infants fed Soja-semp had considerably lower levels.

DISCUSSION

This study shows that it is inadequate to calculate the vitamin E content of food specimens without estimating the γ -tocopherol content. In the Nutramigen formula we found almost as much γ -tocopherol as the α -tocopherol content declared by the manufacturer. Since much of the α -tocopherol in Nutramigen was found to be in the form of acetate-ester, we were unable to determine the total tocopherols in this formula (see Methods). The large content of γ -tocopherol in Nutramigen is due to its high content of corn oil which contains 21.6 mg/100 g α -tocopherol and 76 mg/100 g γ -tocopherol (1). Alfa- and γ -tocopherol differs in their biological activity. Based on the effectiveness of α - and γ -tocopherol to prevent liver necrosis in rats or exudative diathesis in

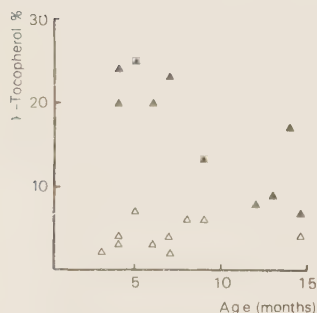


Fig. 1. The relationship between serum γ -tocopherol levels (expressed as % of the total tocopherols) and age in 10 infants fed Soja-semp Δ , and 10 infants fed Nutramigen $\blacktriangle$.

chicks (1), the vitamin E activity of γ -tocopherol is generally considered to be 10% that of α -tocopherol. Thus, if the vitamin E content of the Nutramigen formula had been assessed only as total tocopherols with conventional spectrophotometric methods (8), this would have resulted in an overestimation of the actual vitamin E activity present in the formula.

In the other formula, Soja-semp, γ -tocopherol accounted for about 20% of the total tocopherols. In this formula no α -tocopheryl-acetate was found. Because of the large difference in γ -tocopherol between Nutramigen and Soja-semp, we chose these two formulas to study the effect of γ -tocopherol administration on the serum γ -tocopherol in infants. We did not examine infants fed human milk because of the variability in tocopherol composition of human milk. In an earlier study we found γ -tocopherol to account for 5–32% of the total tocopherols in mature human milk (9).

The serum tocopherol levels presented here are considerably higher than those recently found by Farrel et al. (10) in older children. Of the 20 infants in our study only one had a serum level below 0.5 mg/dl (11.6 μ mol/l). This discrepancy is probably not due to the use of a different laboratory method, but may reflect different nutritional regimens, which is illustrated also by the differences in γ -tocopherol levels between the two groups of infants studied by us. In the Nutramigen group the serum γ -tocopherol levels were nearly 4 times as high as in the Soja-semp group. The difference was most evident in the youngest infants who were almost entirely formula fed (Fig. 1). The γ -tocopherol levels found in these infants also exceeded those found in adults (5, 7). The biological significance of the γ -tocopherol level in serum or plasma is not known. Scott & Desai (11) found that muscular dystrophy in chicks was prevented by similar plasma concentrations of α -, β -, and γ -tocopherol. This suggests that all tocopherols in the tissue may possess the same biopotency. However, if this is true also for humans remains to be showed.

The serum level of the tocopherols, probably also gives an inadequate picture of the tocopherol status. Bieri et al. (1) found a significant tissue accumulation of both α - and γ -tocopherol in rats, but the concentration varied considerably in various organs, adipose tissue accumulating most tocopherols and plasma least.

Our study demonstrates that γ -tocopherol is present in substantial amounts in the serum of infants fed large amounts of it. For this reason it seems warranted to study also serum γ -tocopherol in nutritional surveys, as the serum level of γ -tocopherol reflects the composition of the consumed vitamin E and thus also the intake of vegetable oils rich in γ -tocopherol.

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